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Differentiation-Dependent Expression of E1^E4 Proteins in Cell Lines Maintaining Episomes of Human Papillomavirus Type 31b

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The life cycle of human papillomaviruses (HPVs) is dependent on epithelial differentiation. Among the viral proteins expressed in differentiated epithelial cells are the viral capsid proteins, L1 and L2, as well as the E1^E4 fusion proteins. In this study, the expression and intracellular localization of the E1^E4 proteins of HPV type 31b were examined in both monolayer and raft cultures of the CIN-612 cell line which maintains episomal copies of HPV-31b. In this cell line, a high level of E1^E4 protein expression was observed in the cytoplasm of a small percentage of cells in monolayer culture. A large increase in E1^E4 protein levels was observed upon stratification of the CIN-612 cell line in raft cultures, with E1^E4 protein expression limited to the uppermost layers of the epithelium. A diffuse, slightly grainy cytoplasmic localization of E1^E4 protein was observed in both monolayer and raft culture systems. Although virion synthesis is entirely dependent upon phorbol ester or synthetic diacylglycerol treatment of raft cultures, E1^E4 expression was observed in both treated and untreated monolayer and raft cultures of the CIN-612 cell line. In monolayer cultures of two simian virus 40-transformed cell lines, cos-7 and MK-6, transiently transfected with an E1^E4 expression vector, the distribution of E1^E4 protein was found to differ substantially from that in the CIN-612 cells. In these cell lines E1^E4 protein was found to exhibit a total collapse into either cytoplasmic inclusion granules in the cos-7 cells or a perinuclear halo-like structure in the MK-6 cell line. The host cell, its differentiation state, and the amount of expression can therefore significantly affect the distribution of the E1^E4 proteins.

The life cycle of papillomaviruses is closely linked to epithelial differentiation. Infection by papillomaviruses occurs into exposed basal cells where viral genomes are established as low copy episomes (*1*). As infected cells migrate from the basal region and undergo differentiation, the amplification of viral genomes and the expression of late genes are induced (*2–4*). Among the late gene products that are expressed in differentiated suprabasal cells are the capsid proteins, L1 and L2, as well as a protein resulting from the fusion of the five N-terminal amino acids of E1, a protein involved in viral replication, and the entire E4 open reading frame (ORF) (*5–8*). Little is known about the mechanism by which expression of E1^E4 proteins are regulated or what the function of these proteins are in the viral life cycle. Some studies have suggested that E1^E4 proteins function to collapse the keratin network in differentiated suprabasal cells in order to facilitate viral egress. This model is based upon transient expression studies in monolayer cultures of transformed keratinocyte cell lines (*9*). With the recent development of *in vitro* systems for duplication of the human papillomavirus (HPV) life cycle (*4, 10*) the role of the E1^E4 proteins can be examined as a function of differentiation.

The levels of E1^E4 expression and the biochemical properties of the E1^E4 protein have been previously studied using biopsy material from *in vivo* lesions. HPV-1 E1^E4 protein has been observed in cutaneous warts by immunohistochemical methods, and various E1^E4 species with molecular weights ranging from 10 to 45 kDa have been observed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (*5–7*). The larger molecular weight proteins have been presumed to be multimeric species. In *Condylomata acuminatum* induced by HPV types 6 and 11, only E1^E4 proteins corresponding to the monomeric molecular weights of 10 to 11 kDa were detected (*11, 12*). In addition, E1^E4 proteins of monomeric size were detected in HPV-11 xenograft cultures of human tissue explants in athymic nude mice (*13*). Staining of E1^E4 proteins was also observed in suprabasal cells of explants from HPV-11 xenograft cultures which had been expanded in raft cultures (*10*).

E1^E4 proteins from HPV-1 have been shown by immunohistochemical methods to localize to the cytoplasmic and nuclear inclusion granules present in the suprabasal cells of cutaneous warts (*5–7*). These inclusion bodies were found in peripheral contact with the cytokeratin tonofilament network and were free of filament contact

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in the nucleus (*14*). In contrast, studies by Sterling *et al.* (*15*) demonstrated that HPV-16 E1^E4 proteins associated with the cytokeratin tonofilament network in cervical keratinocytes, but did not localize to inclusion granules. These differences may be the result of the specific viral types examined or the experimental systems utilized.

Several groups have used transient transfection assays of various E4 and E1^E4 genes in numerous transformed epithelial cell lines to study its function. Doorbar *et al.* reported that overexpression of HPV-16 E1^E4 induced the collapse of the cytokeratin intermediate filament network in transfected CV-1, SVK-14, and HaCaT cells due to a direct association between the endogenous cytokeratins and the exogenous HPV-16 E1^E4 protein (*9*). Similar results have been reported by Rogel-Gaillard *et al.* (*16*) on the collapse of HPV-1 E1^E4 protein into cytoplasmic inclusions in transfected VX2R cells and with HPV-16 E1^E4 in transformed keratinocytes by Roberts *et al.* (*17*). In order to examine the role of the E1^E4 protein in the life cycle of a high risk HPV type, we have utilized the organotypic (raft) culture system for epithelial differentiation (*4, 18*). Using raft cultures of the CIN 612 cell line which stably maintains episomes of HPV-31b, we have previously observed the differentiation-specific amplification of viral DNA (*2*), induction of late gene expression, and virion formation (*4*). In this present study we extend our analysis to include the regulation of E1^E4 protein synthesis and its distribution in raft cultures.

In order to characterize the HPV-31b E1^E4 protein we first generated antisera and constructed expression vectors. The E1^E4 cDNA sequence was amplified by the polymerase chain reaction from a cDNA isolated from CIN-612 cells (*19*). This E1^E4 cDNA sequence was then cloned into the simian virus 40 (SV40) expression vector pSG5 (*20*) to produce pSG31–E1^E4. In addition, this fragment was subcloned into pEX-1 (*21*) to produce a β-galactosidase–E1^E4 fusion protein from the plasmid pEX31–E1^E4. This protein was then purified by size-exclusion chromatography and used to produce polyclonal antibodies. E1^E4 antisera was subsequently affinity-purified against a bacterially expressed glutathione *S*-transferase–E1^E4 fusion protein (*22*).

We first examined synthesis of HPV-31b E1^E4 proteins using transient transfection assays in SV40-immortalized cell lines. Other investigators have used this methodology to observe a collapse of cytokeratins upon expression of HPV-16 E1^E4 proteins (*9*). For our study, pSG31–E1^E4 was electroporated into either cos-7 cells or MK-6 cells (*23*), an SV40-immortalized human foreskin keratinocyte cell line, and total cell extracts isolated at 36 hr postelectroporation. Equal amounts of protein extracts from mock (pSG5)- and pSG31-E1^E4-transfected cos-7 and MK-6 cells and CIN-612 cultures were subjected to SDS–PAGE, transferred to a PVDF membrane (Millipore), and immunoblotted with affinity-purified anti-E1^E4 se-

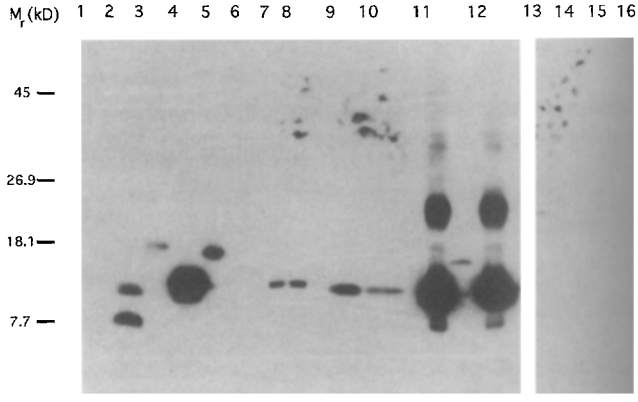


FIG. 1. Western blot analysis of E1^E4 expression in transfected cos-7 and MK-6 cells and in CIN-612 cultures. Lanes 1 and 3 contain extracts from mock (pSG5)-transfected cos-7 and MK-6 cells, respectively, while lanes 2 and 4 contain the pSG31–E1^E4-transfected cell extracts of the cos-7 and MK-6 cells, respectively. Lanes 1–4 represent the urea-soluble fractions of these isolates, as no E1^E4 was detected in the RIPA-soluble fractions. Lanes 5–8 contain extracts from monolayer cultures of CIN-612 cells. Lanes 5 and 6 represent RIPA-soluble fractions, while lanes 7 and 8 contain the urea-soluble extracts. Lanes 5 and 7 are C8:0-treated samples; lanes 6 and 8 represent the untreated sample. Similarly, lanes 9–12 represent the raft-cultured CIN-612 cells. Lanes 9 and 10 contain the RIPA-soluble fractions, and lanes 11 and 12 the urea-solubilized extract. Lanes 9 and 11 represent the C8:0-treated sample, while lanes 10 and 12 contain extract from the untreated raft culture. Lanes 13 and 14 contain the urea-soluble fraction of extracts isolated from HaCaT cell raft cultures, treated and untreated with C8:0, respectively. Lanes 15 and 16 contain the RIPA-soluble and urea-soluble fractions, respectively, of extracts isolated from a C8:0-treated MK-6 raft culture. The spots seen in lanes 3 and 5 are nonspecific and due to ECL processing. No similar spots were observed in other analyses. *Methods.* Cos-7 cells were grown in D-MEM (JRH Biosciences) supplemented with 10% heat-inactivated calf serum (Gibco-BRL Life Technologies). MK-6 and CIN-612 monolayer cultures were grown in E media (*4*). Raft cultures were prepared as previously described (*4, 18*), 1,2-dioctanoyl-*sn*-glycerol (C8:0) (Sigma) was maintained in the medium at a concentration of 10 μM. Electroporations were performed at room temperature in 1 ml PBS at 1000 V and 25 μF, with 10 μg circular plasmid DNA and 1×10^6 cells, using a Bio-Rad Gene-Pulser apparatus. Monolayer cells were harvested by scraping with a rubber policeman, and raft cultures were isolated by scraping the grown tissue from the underlying collagen matrix with a scalpel. All samples were mechanically homogenized (by a tissue homogenizer for the raft cultures or a 23-gauge needle for the monolayers) on ice in RIPA–SDS buffer (150 m*M* NaCl, 50 m*M* Tris–HCl (pH 8.0), 5 m*M* Na_2EDTA, 100 m*M* NaF, 200 μM Na_3VO_4 (*ortho*), 1.0% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 1.0% (wt/vol) deoxycholic acid, 1 m*M* DTT, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 m*M* benzamidine–HCl) and incubated for 1 hr at 4°. After centrifugation at 14,000 *g* for 15 min, the insoluble pellet was resuspended in 8 *M* urea, 10% β-mercaptoethanol, 2 m*M* PMSF, and incubated at 37° for 30 min. The insoluble debris was removed by centrifugation as above. Equal amounts (50 μg) of total cellular protein were then electrophoresed on a 12.5% SDS–polyacrylamide gel, transferred to an Immobilon PVDF membrane (Millipore) in a Bio-Rad Trans-Blot cell overnight, and incubated with α-E1^E4-P at a concentration of 0.1 μg/ml in 1% (wt/vol) nonfat dry milk, 0.1% (vol/vol) Tween 20 in Tris-buffered saline. The blot was then developed using the ECL-Western kit per the manufacturer's instructions (Amersham).

rum (α-E1^E4-P) (Fig. 1). Two species of HPV-31b E1^E4 protein were detected in the transfected cos-7 extract with molecular weights of 11 and 8 kDa (lane 2). The smaller species most likely represents a proteolytic degradation product. On the other hand, extracts from electroporated MK-6 cells were found to contain only a single species of the E1^E4 protein, which migrated at 11 kDa (lane 4). No evidence for any multimerization of E1^E4 was seen in either of these electroporated cell types. As seen in Fig. 1, low levels of E1^E4 protein were also observed in monolayer cultures of CIN-612 cells (lanes 5–8), with a molecular weight similar to that seen in the transfected MK-6 cells. No specific hybridization was detected in control extracts of mock-transfected cos-7 or MK-6 cells (lanes 1 and 3).

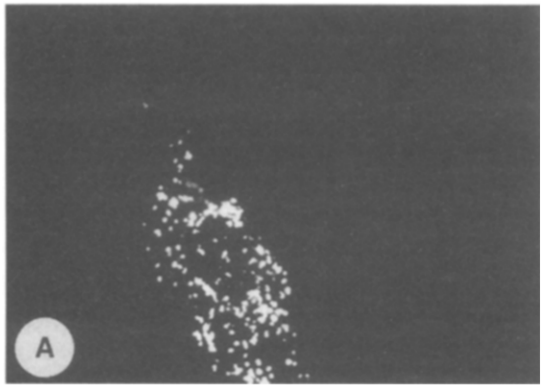
Transcripts encompassing the E1^E4 region of HPV-31b have previously been observed in both monolayer and raft cultures of CIN-612 cells (*19*). In monolayer cultures, the major viral transcripts initiate upstream of the E6 ORF at nucleotide 97 and encode multiple viral ORFs. The predominant message encodes E6*, E7, E1^E4, and E5. Upon differentiation, a novel promoter in the E7 ORF at nucleotide 742 is activated and the corresponding transcript encodes E1^E4 and E5. To examine if synthesis of E1^E4 proteins increased upon differentiation we next compared the level of protein synthesis in monolayer cultures to that seen in rafts.

As shown in Fig. 1, the level of E1^E4 protein in CIN-612 cells was increased dramatically upon differentiation in raft cultures (lanes 9–12). In addition, a more complex pattern of E1^E4 protein species was observed in raft cultures than that seen in the monolayer cells. The predominant form of E1^E4 synthesized upon differentiation migrated at 11 kDa, consistent with the monomeric size of the protein observed in monolayer cultures. However, additional forms of approximately 18 kDa, as well as several possible multimeric species near 22, 33, and 44 kDa were observed. These higher molecular weight forms were observed to migrate as doublets. Some faster migrating species were also detected which possibly represent proteolytic fragments. No specific hybridization was observed in control extracts of HaCaT or MK-6 cell raft cultures (lanes 13–16). The multimeric species of E1^E4 observed in raft cultures are similar to those reported in analyses of biopsy material from HPV-1-infected warts (*5–7*).

In previous studies, we reported that the addition of phorbol esters or synthetic diacylglycerols (DAGs) to raft cultures of CIN-612 cells was required for the induction of capsid protein synthesis and virion production (*4*). Since E1^E4 synthesis was also activated upon differentiation, it was possible that synthesis of this protein was similarly responsive to such treatment. However, only a slight increase in the level of E1^E4 synthesis was observed following treatment of raft cultures of CIN-612 cells with 1,2-dioctanoyl-*sn*-glycerol (C8:0) (Sigma), a synthetic DAG (lanes 9–12). This indicates that E1^E4 protein synthesis is most likely regulated by a different mechanism than are the capsid proteins, L1 and L2. This is consistent with our studies on HPV 31b expression indicating that two distinct transcripts encode E1^E4/E5 and E1^E4/L1 (M. Hummel *et al.*, manuscript in preparation). Similar observations have been made with transcripts isolated from biopsies of HPV 11-infected tissue (*8*). No detectable increase in E1^E4 protein synthesis was seen in the monolayer culture following C8:0 treatment (lanes 5–8).

We next sought to examine the subcellular localization of E1^E4 in the various cell lines. For these studies, indirect immunofluorescence experiments were performed using α-E1^E4-P primary antisera and a Texas red-conjugated donkey anti-rabbit Ig secondary antibody (Amersham) (Fig. 2). We observed that in transfected cos-7 cells at 36 hr postelectroporation, E1^E4 protein was distributed in a punctate pattern throughout the cytoplasm (Fig. 2A), as has previously been described for HPV-1 protein (*7*). A small number of electroporated cos-7 cells also exhibited a slight amount of punctate nuclear staining (Fig. 2A). A different subcellular localization was observed in the MK-6 transfectants, where E1^E4 was found to localize to the perinuclear region (Fig. 2C). Interestingly, the localization pattern of E1^E4 changed as a function of time following electroporation. At early time points (up to 24 hr) E1^E4 exhibited an intermediate filament-like distribution, and by 48 hr postelectroporation this structure had collapsed into large inclusion granules located adjacent to the nuclei (data not shown). These time-dependent distributions are similar to those previously reported in transient expression assays of E1^E4 proteins in transformed epithelial cell lines (*9*) and may represent changes due to high level expression from transfected vectors.

The distribution of E1^E4 proteins in monolayer cultures of CIN-612 cells was found to be significantly different from that seen in the transfected cells. In CIN-612 monolayers, the E1^E4 protein appeared as a generally diffuse, slightly grainy cytoplasmic signal in a small minority of cells (approximately 2%) (Figs. 2E and 2G). However, expression in this small number of cells was high, as the intensity of the secondary antibody's excited fluorochrome was nearly equal to that seen in the transiently transfected MK-6 cells (Fig. 2C). The presence of E1^E4 in only a small percentage of monolayer CIN-612 cells may be due either to restricted accessibility of antibodies to the antigen or to the existence of a low number of cells which have spontaneously activated late functions. Interestingly, using standard reflected light microscopy, the E1^E4 protein appeared to be present not only in the cytoplasm, but also at low levels in the nuclei of monolayer CIN-612 cells (data not shown). However, using confocal microscopy, it was apparent that E1^E4 was strictly cytoplasmic. Studies by Burnett and colleagues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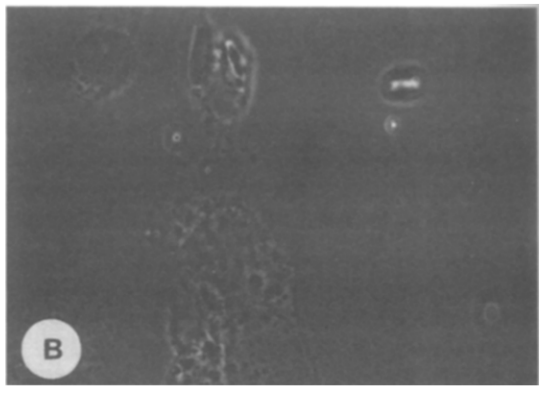
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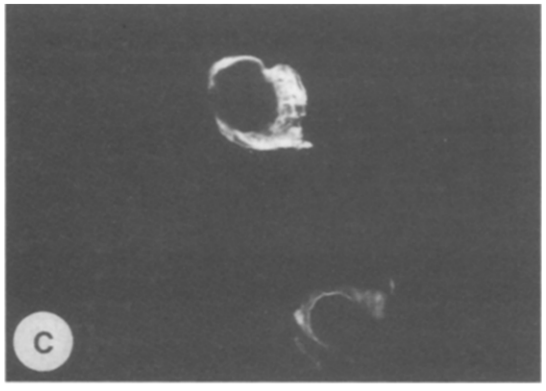
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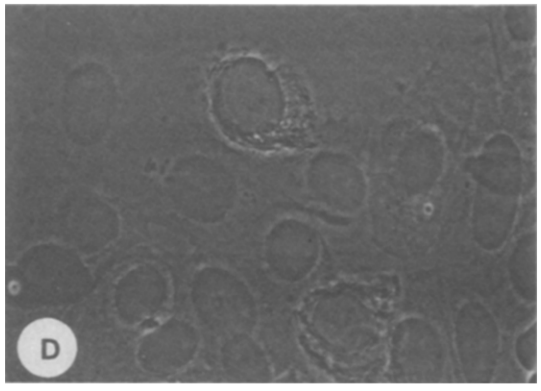
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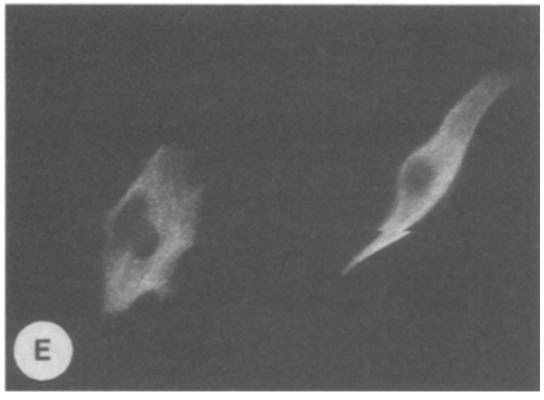
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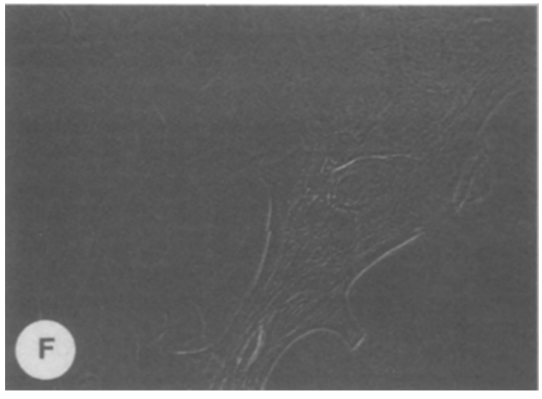
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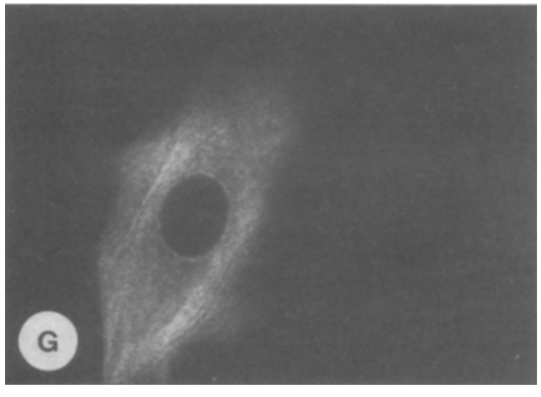
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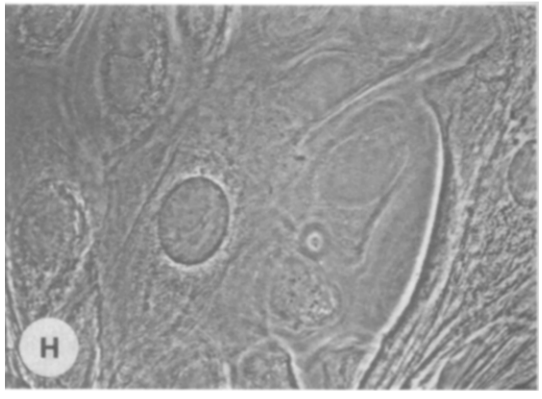
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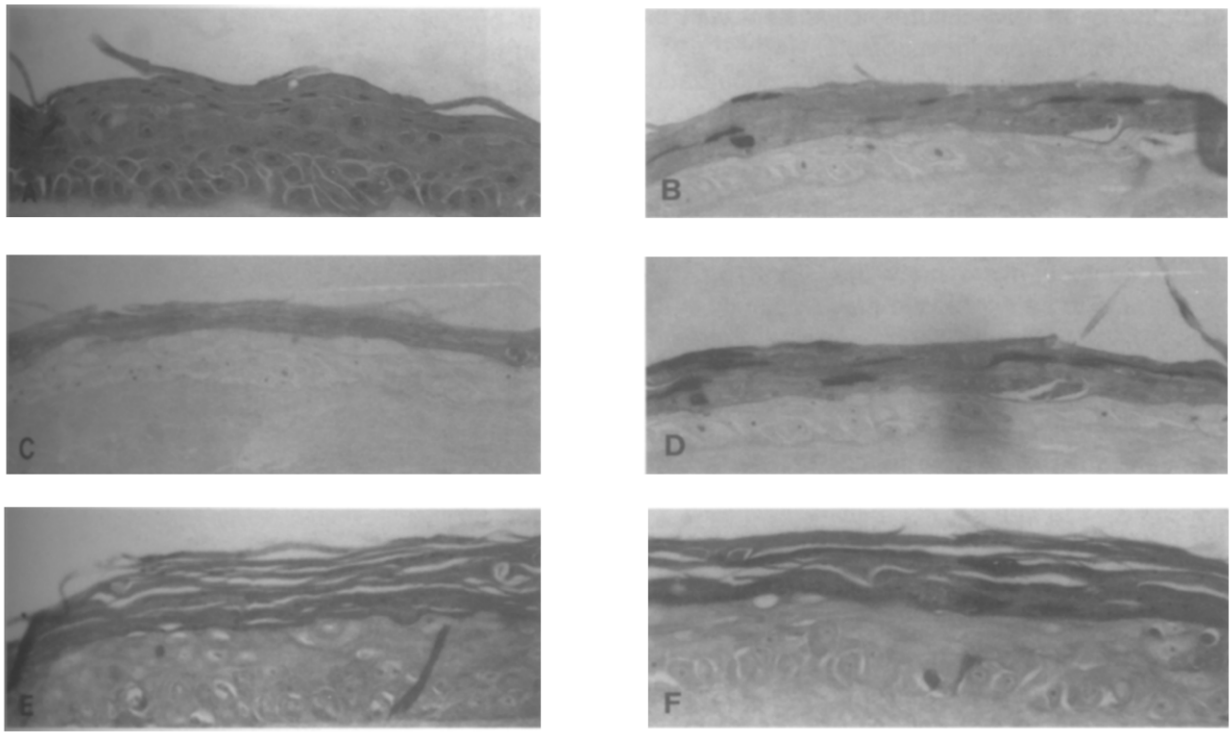

FIG. 3. Immunohistochemical localization of E1^E4 and L1 proteins in a C8:0-treated, CIN-612 raft culture utilizing a peroxidase detection reaction (Vectastain Elite ABC detection kit, Vector Laboratories). A shows an H and E stain of a representative section of the culture. B–D are photomicrographs of serial sections of the same C8:0-treated culture. E and F are from an untreated raft culture. B and E were incubated with anti-HPV-16 L1 antisera, C was incubated with a purified rabbit anti-mouse antibody as a negative control, while D and F were incubated with α-E1^E4-P. All images were photographed at 436× magnification. *Methods.* Raft cultures, grown as described in the legend to Fig. 1 were fixed in 4% (wt/vol) paraformaldehyde in PBS, paraffin-embedded, and sectioned onto poly-L-lysine-coated slides. Slides were baked at 55° for 1 hr, hydrated through a graded series of ethanol washes, treated with 3% (vol/vol) H_2O_2 in PBS for 30 sec to quench endogenous peroxidase activity and with 0.1 mg/ml pronase E (Sigma) in PBS at 37° for 10 sec to liberate antigenic epitopes. Sections were blocked and incubated with primary antisera as described in the legend to Fig. 2 and then blocked again and treated with secondary antisera and detection reagents per the manufacturer's instructions. Images were photographed using an Olympus BHS system microscope.

(*24*) have demonstrated that late papillomavirus functions such as genome amplification can be induced following incubation of BPV-1-transformed cells at confluence for extended periods of time. However, in our studies activation of E1^E4 appeared spontaneously in monolayer cultures and did not require incubation at confluence.

We next examined the distribution of E1^E4 proteins in stratified raft cultures using a peroxidase staining method. Serial sections of raft-cultured CIN-612 cells were incubated with either α-E1^E4-P or HPV-16 L1 antisera (a kind gift from J. Schiller (*25*)), and typical results are shown in Fig. 3. This analysis allowed us to examine if there was a relationship between L1 and E1^E4 protein synthesis (Figs. 3B, 3D–3F). Synthesis of E1^E4 proteins was observed in individual cells distributed throughout the upper portion of the epithellium. E1^E4 was observed primarily in the spinosum and granulosum of the differentiated epithelium, with a small amount extending into the cornified layer of the tissue. Furthermore, expression of E1^E4 was detected in numerous cells which were not positive for L1. However, most cells that were positive for L1 also synthesized E1^E4. While L1 synthesis was strictly dependent on C8:0 treatment (Figs. 3B and 3E), E1^E4 exhibited a similar distribution in both treated and untreated rafts (Figs. 3D and 3F). However, the level of E1^E4 expression and the number of positively staining cells appeared to

FIG. 2. Indirect immunofluorescent laser-scanning confocal microscopy of E1^E4 in cos-7, MK-6, and CIN-612 monolayer cultures. A, C, E, and G are indirect immunofluorescent images, while B, D, F, and H represent the phase-contrast images of the same fields. A and B show the pSG31-E1^E4-transfected cos-7 cells, while C and D contain the transfected MK-6 cells. E–H show the CIN-612 monolayer culture. G represents a higher magnification of one cell seen in E. A–D, G, and H were photographed at 775× magnification, while E and F were photographed at 388×. *Methods.* Cells were grown on Permanox 2-well chamber slides (Nunc) as described in the legend to Fig. 1. Cells were fixed following the periodate–lysine–paraformaldehyde fixation technique (*30*). After permeabalization with 0.25% (vol/vol) Triton X-100 in phosphate-buffered saline (PBS), slides were preincubated with 20% normal goat serum (NGS) (Sigma) in PBS and then incubated with 1 μg/ml α-E1^E4-P. Following washes with 0.1% (vol/vol) NP-40 in PBS, samples were blocked again and incubated with Texas red-conjugated donkey anti-rabbit Ig antiserum (Amersham) at a dilution of 1:250. Slides were then washed, air-dried, and coverslipped using 1 mg/ml 1,4-diazabicyclo-[2.2.2]octane (Sigma) in 90% glycerol/10% PBS as an antioxidant mounting medium. Images were photographed with a Zeiss LSM-10 microscope.

slightly increase in treated cultures, consistent with the levels observed in Western immunoblots.

To examine in more detail the intracellular localization of the E1^E4 protein in differentiated CIN-612 cells, experiments were initiated using fluorescent labelling methods. These assays provide a more accurate view of the subcellular localization of proteins than the peroxidase staining method used in Fig. 3. Using indirect immunofluorescent confocal microscopy, the HPV-31b E1^E4 protein was found to be expressed predominantly in the suprabasal cells of both the C8:0-treated and -untreated raft cultures. In these positive cells it was found exclusively in the cytoplasm (Fig. 4). While E1^E4 was generally distributed in a diffuse manner throughout the cytoplasm in positive-staining cells, the pattern appeared punctate, suggesting that focal concentrations of E1^E4 were present. The E1^E4 protein appeared to contact the nuclear membrane in these cells, and extend to the cellular periphery. No collapse of the HPV-31b E1^E4 protein into large cytoplasmic (or nuclear) inclusion bodies was observed in either treated or untreated cultures, in contrast to studies with the HPV-1 E1^E4 protein (*5–7*). Consistent with our observations of E1^E4 synthesis in a small number of monolayer cells, an occasional cell (less than 1%) in the basal layer of the stratified cultures also stained positive for E1^E4 (data not shown).

The difference in the subcellular localization observed between the E1^E4 proteins of HPV types 1, 16, and 31b may be due to the tissue specificity of the different viral types or differences in the primary structure of the E1^E4 proteins. HPV-16 and -31b target mucosal tissue, whereas HPV-1 infects the cutaneous epithelium of the palmar and plantar surfaces. Therefore, the differences observed could be due to variations in the tissue-specific expression of the cytokeratins with which E1^E4 may interact (*26*). Alternatively, differences may exist in the physical and structural characteristics of the E1^E4 proteins encoded by the mucosal and cutaneous viruses, since the E4 ORFs are not highly conserved among viral types (*27*). The presence of E1^E4-containing cytoplasmic inclusion granules in HPV-1-infected warts is in agreement with the observation that transiently expressed E1^E4 proteins are capable of inducing intermediate filament collapse in transformed epithelial cells. However, our data and that of Sterling *et al.* are consistent with the fact that large inclusion granules are not generally found in HPV-infected mucosal tissue. While the presence of grainy E1^E4 staining in HPV-31b-containing raft cultures may indicate that this protein is capable of inducing nucleation, it is our belief that the E1^E4 proteins of the mucosal HPVs do not induce inclusion body formation in the natural host cell. Studies by Brown *et al.* (*28*) examining HPV 11 xenografts in nude mice indicated that E1^E4 was predominantly cytoplasmic and appeared to be membrane associated. In our studies, E1^E4 proteins were found to be distributed throughout the cytoplasm. These differences may be the result of variations in fixation methodologies, differences in HPV type, or our use of confocal microscopy to characterize protein distributions.

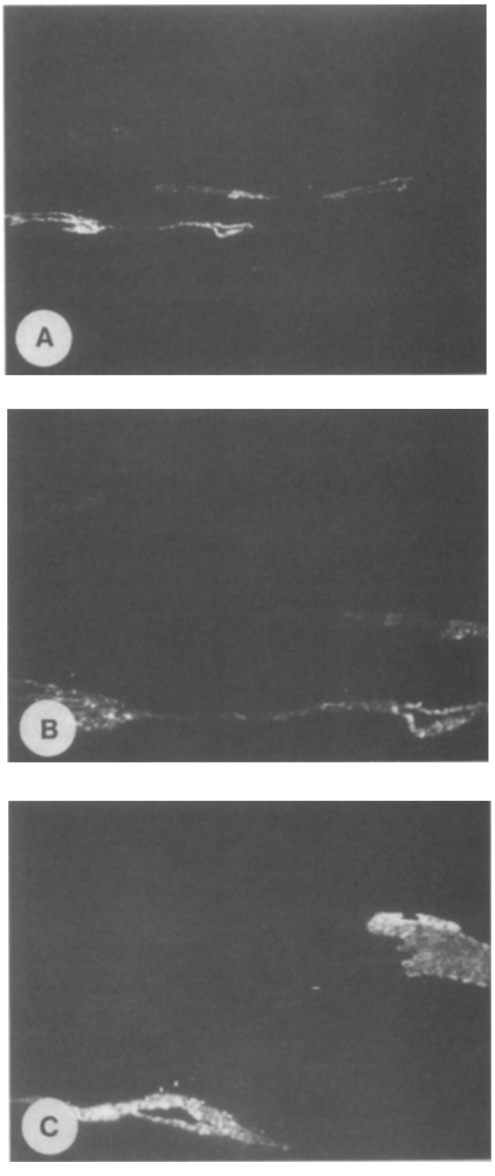

FIG. 4. Indirect immunofluorescent laser-scanning confocal microscopy of E1^E4 protein in raft cultures of CIN-612 cells. A shows a representative section of an untreated raft culture incubated with α-E1^E4-P and photographed at 440×. B is a higher magnification (1104×) image of the left side of A. C is a 1840× image of a section of a C8:0-treated raft culture incubated with α-E1^E4-P. *Methods.* Raft cultures were prepared as described in the legend to Fig. 3. Slides were baked, hydrated, and protease-treated as described in the legend to Fig. 3. They were then blocked and incubated with antisera as described in the legend to Fig. 2. The slides were then dehydrated through the alcohol series, air dried, and coverslipped and photographed as described in the legend to Fig. 2.

Our data also demonstrate the importance of studying E1^E4 activity in the correct culture system. We observed dramatic differences in E1^E4 subcellular localization

between HPV-31b episome-containing cells and transiently transfected, SV40-transformed cells. It has been reported that T-antigen expression may disrupt keratin expression (*29*), and this could be a major factor in the observed collapse of HPV types 16 and 31b E1^E4 protein in transfected, SV40-immortalized cell lines. Overexpression of E1^E4 in these cells may induce a default pathway which includes collapse of the subcellular, keratin-derived architecture. Perhaps the synthesis of viral gene products in HPV-31b episome-containing basal cells may prime the cellular environment for the correct targeting of the E1^E4 gene product. Further functional studies on the role of the E1^E4 protein may help to further define a role for this protein in the pathogenesis of the high risk human papillomaviruses.

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